

Short Communication

Oxygen Fixation into Hydroxyproline in Etiolated Maize Seedlings¹

VERIFICATION BY TANDEM MASS SPECTROMETRY

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ABSTRACT

Etiolated maize (*Zea mays* L.) seedlings were grown in the dark for 5 days in an atmosphere enriched with 10.0 atom % ¹⁸O₂. Hydroxyproline was isolated from root and shoot tissues, purified, and methylated. It was not possible to determine ¹⁸O incorporation into hydroxyproline by conventional mass spectrometry because the final product was not sufficiently pure. The final product was analyzed successfully by tandem mass spectrometry. The ¹⁸O content of the hydroxyl oxygen atom was 10 ± 0.7 atom %. This result demonstrates that the hydroxyl oxygen atom in hydroxyproline was derived exclusively from molecular oxygen.

atom in hydroxyproline is derived from O₂ and not from water.

The recent development of tandem mass spectrometry (MS/MS) and the availability of the instrument in one of our laboratories (R. A. Y.) persuaded us to reexamine O₂ fixation into hydroxyproline in etiolated maize seedlings. In MS/MS, a sample mixture is ionized and the molecular ion of one component is separated by a first stage of mass analysis; that ion is then fragmented, and identified in a subsequent stage of mass analysis (6, 7). In terms of the analysis for ¹⁸O-labeled hydroxyproline, the first mass analyzer is employed to separate the labeled and unlabeled compounds; the other mass analyzer provides a mass spectrum for confirmation of the separated compounds. To our knowledge, this is the first report of the use of MS/MS for the investigation of stable isotope incorporation in living systems.

MATERIALS AND METHODS

It is generally accepted that atmospheric O₂ is the source of the hydroxyl oxygen atom in the imino acid hydroxyproline in plants (1, 2). This view has been supported by the following isotopic O₂ experiments. A suspension of sycamore cells was grown in an atmosphere enriched with ¹⁸O₂ in one study (3). Hydroxyproline isolated from the cells was purified. Details of MS analysis of hydroxyproline were not described but it was reported that ¹⁸O enrichment was in good agreement with the theoretically calculated value, and it was concluded that the hydroxyl oxygen atom in hydroxyproline in sycamore cells is derived exclusively by O₂ fixation. In a second study, maize grains were germinated in sealed Erlenmeyer flasks, and grown for several days in the dark in an atmosphere enriched with ¹⁸O₂ (4). Hydroxyproline was isolated from roots and shoots, purified, and pyrolyzed to CO₂. MS analysis of CO₂ showed that ¹⁸O enrichment was 24% of the theoretically calculated value in one experiment and 38% in another experiment; a suggestion forwarded to account for the low ¹⁸O enrichment was the possible existence of impurities in recovered hydroxyproline. To eliminate the possibility of incorporation of H₂¹⁸O derived from respiration into hydroxyproline, a control experiment was carried out. Seedlings were grown in water enriched with ¹⁸O; the atmosphere contained O₂ of natural abundance. Hydroxyproline obtained from these seedlings was found to have the natural abundance of ¹⁸O. It was concluded that the hydroxyl oxygen

Plant Material and Growth Conditions. Maize grains (*Zea mays* L., Wf9 × 38-11, fertile version) were soaked for 6 h in water. Then they were sown in vermiculite in a glass chamber whose volume was approximately 1000 cm³. The vermiculite was wetted with sufficient water at the start of the growth period so that further additions were not needed. To absorb respiratory CO₂, a glass jar containing about 50 ml 40% NaOH was placed upright inside the chamber; the contents were stirred continuously (with a magnetic stirrer) during the growth period to prevent the formation of a surface layer of solid Na₂CO₃. The glass chamber, sealed with a large rubber stopper fitted with a glass tubing that was sealed with rubber tubing and a clamp, was covered with black cloth throughout the growth period.

Labeled O₂ was prepared by electrolysis of ¹⁸O-enriched water reported to contain 10.0 atom % oxygen-18 (Bio-Rad Laboratories). A gas mixture of labeled O₂ and N₂ (21:79 v/v) was introduced into the glass chamber by evacuating the chamber with a water pump and filling to 1 atm pressure. Thereafter, O₂ absorbed by the growing seedlings was replaced every 6 to 8 h with pure O₂ containing 10.0 atom % oxygen-18. At the end of the growth period (5.0 d from the time of immersion of grains into water), the seedlings were harvested. Only the shoot-root tissue (4-8 cm long; 37-40 g fresh weight in each of separate batches) was recovered and used for the isolation of newly formed hydroxyproline because preliminary investigation showed that cotyledons and endosperm contained relatively large quantities of preformed hydroxyproline.

Maize grains were also grown in wetted vermiculite in trays that were held in a darkened closet and exposed to laboratory

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air. Seedlings were harvested as described above and used in preliminary experiments.

Isolation and Purification of Hydroxyproline. Although the isolation of hydroxyproline and its purification were generally patterned after previously published work (4), several substantive changes were made, so the procedure warrants description here. Frozen and thawed plant tissue (25 g fresh weight) was pulverized in liquid N₂ in a mortar with pestle. The ground tissue was boiled in 95% ethanol for 30 min and filtered. The extract was partitioned with petroleum ether in a separatory funnel; the ether fraction was discarded. The ethanol fraction was evaporated to dryness in a rotary evaporator. (In this and all subsequent operations involving the use of the rotary evaporator, the temperature did not exceed 55°C.) Then the ground tissue and 6 N HCl were added to the ethanol residue, and the mixture was hydrolyzed by boiling for 24 h under a water-cooled reflux condenser. (This treatment did not result in exchange of the hydroxyl oxygen atom with water of the medium [4].) The insoluble residue was removed by filtration. The hydrolysate was evaporated to dryness in a rotary evaporator; water and 1 g charcoal were added and the mixture was stirred gently for 2 h. The carbon was removed by filtration, washed well with water, and discarded. The water extract containing hydroxyproline was reduced in volume on a rotary evaporator to about 25 ml, and placed on a column (20 x 1.5 cm) of Amberlite IR-120 (medium porosity) cation exchange resin, prepared by washing first with 6 N HCl and then with water. Now the column was washed with water, eluted with 200 ml 2 N NH₄OH, and the effluent evaporated to dryness on a rotary evaporator. Nitrous acid (prepared immediately prior to use by mixing 1 part of a solution of 30% NaNO₂ with 2 parts of 9 N HCl) was added to deaminate primary α -amino acids. This step was carried out at room temperature; the mixture remained at room temperature overnight and then was concentrated on a rotary evaporator. The precipitated NaCl was removed by filtration, washed with cold HCl, and filtered again; the process was repeated three times. Then the remaining solution was evaporated to dryness on the rotary evaporator. After adding about 25 ml water, the solution was placed on an Amberlite IR-120(H⁺) column, and washed and eluted as above.

The effluent was evaporated to dryness on a rotary evaporator, dissolved in about 0.5 ml ethanol, spotted on six thin layer cellulose plates, and developed in a mixture of normal butanol, acetic acid, and water (63:27:10). (In this and subsequent TLC, the location of hydroxyproline was determined by spotting and developing authentic unlabeled hydroxyproline on a separate plate. This plate was sprayed with ninhydrin solution and heated in an oven at 100°C for several min. [Note: In our experience, this heat treatment of plates that held ¹⁸O-labeled hydroxyproline resulted in loss of the label.]) Hydroxyproline spots were scraped off, eluted with 95% ethanol, and re-chromatographed three times on silica gel plates, using the same solvent mixture as above. The technique of multiple development was used (5). That is, in the first run on silica gel (three plates), the plates were dried after development and reinserted into the developing chamber for redevelopment; this was repeated three times. The second run (two plates) also involved drying and redeveloping the plates three times. In the third run (one plate), the plate was dried and redeveloped twice.

After hydroxyproline was recovered by scraping the spots, eluting with 95% ethanol, and evaporating to dryness on a rotary evaporator, about 5 ml of glass-distilled methanol was added to the flask. Hydroxyproline was methylated by passing dry HCl (generated by dropwise addition of H₂SO₄ on dry NaCl in an all-glass apparatus) into the methanol solution of hydroxyproline for several minutes. It was these samples, brought to dryness by evaporation in a stream of N₂, that were analyzed mass spectrometrically.

Mass Spectrometric Analysis. In preliminary experiments methylated hydroxyproline that was isolated and purified from seedlings, as described above, was analyzed for hydroxyproline by MS in one of our laboratories (B. D. A.). Samples were introduced directly by distillation (over a range of 25–310°C) into the electron impact (70 electron volts) ion source of a Hewlett-Packard 5985 quadrupole mass spectrometer. In the remainder of the experiments, isolated, purified, and methylated hydroxyproline was analyzed by tandem mass spectrometry (MS/MS) in one of our laboratories (R. A. Y.). Samples in the range of 10 to 100 ng were introduced by probe (heated ballistically from 30–250°C) directly into the chemical ionization source (methane, 1.0 torr) of a Finnigan MAT triple stage quadrupole mass spectrometer. The protonated molecular ion (mass/charge [*m/z*] 146 for ¹⁶O or *m/z* 148 for ¹⁸O) fragmented by collision with N₂ (1.0 × 10⁻³ torr, 20 electron volts ion energy) in the center quadrupole, and the resulting daughter ion spectrum was obtained by scanning the third quadrupole. The data were acquired and processed by the Finnigan INCOS data system. Quantitation of the degree of isotope incorporation into the hydroxyl group of hydroxyproline was based on the relative intensities for the loss of CH₃COOH from the protonated molecular ion (monitoring the *m/z* 146 → *m/z* 86 reaction for ¹⁶O and *m/z* 148 → *m/z* 88 reaction for ¹⁸O-labeled hydroxyproline). The values reported here are based on at least five analyses of each sample, with at least 100 scans acquired in each analysis (unpublished data).

RESULTS AND DISCUSSION

In preliminary experiments, several samples of hydroxyproline were isolated, purified, and methylated from *Z. mays* seedlings that had been grown in an atmosphere containing the natural abundance (0.2 atom %) of oxygen-18. The identity of the compound was confirmed by TLC comparisons with that of authentic hydroxyproline. MS analyses of these samples showed a complex mixture of biochemicals. The interfering compounds would have made calculation of the degree of oxygen-18 incorporation into hydroxyproline impossible.

Tandem mass spectrometry was therefore employed. The daughter spectrum of the protonated molecular ion of methylated hydroxyproline (*m/z* 146) in the sample obtained from seedlings grown in an atmosphere containing the natural abundance of ¹⁸O matched that of authentic hydroxyproline. The daughter ion spectrum of *m/z* 148 in the sample lacked adequate sensitivity to detect the naturally occurring ¹⁸O hydroxyproline at the expected 0.2 relative intensity arising from the natural abundance of ¹⁸O. Methylated samples obtained from seedlings grown in 10.0 atom % ¹⁸O₂ showed 10.0 ± 0.7% incorporation of ¹⁸O in the hydroxyl group of hydroxyproline. This confirms that the hydroxyl oxygen atom is derived exclusively from molecular oxygen.

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